

HUMAN B CELL DIFFERENTIATION

II. Pokeweed Mitogen-responsive B Cells Belong to  
a Surface Immunoglobulin D-negative Subpopulation\*

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Accidental inoculation of a root extract of *Phytolacca americana* (pokeweed) was noted in 1965 to induce a wave of lymphoblasts and plasma cells in the circulation (1), and mitogenic extracts from this plant have proven very useful for the in vitro study of human B cell differentiation (2). The responsive B cells in blood are slowly acquired during infancy (3), require non-major histocompatibility complex-(MHC) restricted T cell helper factors for activation (2, 4), express surface IgM more often than IgG and IgA, and are easily and selectively inhibited by antibodies to these immunoglobulin (Ig) isotypes (5). In contrast, anti- $\delta$  antisera have been reported to have variable effects on pokeweed mitogen (PWM)-induced differentiation of B cells (6, 7). Other studies have suggested that PWM-responsive cells are of relatively low buoyant density (8) and lack the receptors for mouse erythrocytes found on many human B cells (9).

In the present studies, we examined the expression of IgD on PWM-responsive B cells and correlated these features with cell density, size, and capacity to bind mouse erythrocytes. The results suggest that this is a distinct subpopulation of B cells that are preactivated in vivo.

Materials and Methods

*Cell Preparation and Culture.* Human mononuclear cells (MNC) obtained from heparinized venous blood of healthy donors by Ficoll-Hypaque gradient centrifugation were incubated at 37°C for 1 h in a plastic dish. Nonadherent cells were adjusted to the concentration of  $1 \times 10^6$  cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) 2 mM L-glutamine, 50  $\mu$ g/ml gentamicin, and  $5 \times 10^{-5}$  M 2-mercaptoethanol. Cultures were established in flat-bottomed microplates (Costar, Data Packaging, Cambridge, MA) with each well containing 0.2 ml of the cell suspension and an optimum concentration of pokeweed mitogen (5  $\mu$ l/ml of PWM; Gibco) and held at 37°C for 7 d in a humid atmosphere of 5% CO<sub>2</sub> and 95% air.

*Monoclonal Anti- $\delta$  Antibody.* This mouse monoclonal hybridoma antibody ( $\delta$ -TA4-1,  $\gamma$ 3 $\kappa$ ) was prepared by Dr. H. Kubagawa and Dr. J. F. Kearney (University of Alabama in Birmingham, Birmingham, AL), and its specificity for human  $\delta$  chain was established by enzyme-linked

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immunoabsorbent assays and immunofluorescence staining of a panel of myeloma cells (to be published elsewhere). The concentration of the monoclonal anti- $\delta$  antibody, purified from mouse ascites by affinity column chromatography, was estimated from the optical absorbance at 280 m $\mu$  ( $E_{280}^{1\%} = 14.0$ ).

**Depletion of Surface (s) IgD<sup>+</sup> Lymphocytes.** IgD-bearing cells were selectively depleted from blood MNC preparations by the "panning" method or using a fluorescence-activated cell sorter (FACS), as described previously (5). Briefly,  $1-1.5 \times 10^7$  MNC were treated either with monoclonal anti- $\delta$  antibody (200  $\mu$ g/ml) in Dulbecco's phosphate-buffered saline (PBS) or with PBS alone at 4°C for 20 min. After washing, the cells were incubated for 70 min at 4°C in plastic dishes previously coated with affinity-purified rabbit anti-mouse IgG antibody. The nonadherent cells, after three or more depletion cycles, were harvested and resuspended in RPMI 1640 culture medium. A FACS IV (B-D FACS systems, Becton, Dickinson & Co., Sunnyvale, CA) was used to remove sIgD<sup>+</sup> cells in other experiments. Blood MNC were incubated with monoclonal anti- $\delta$  antibody (200  $\mu$ g/ml) and stained with fluorescein-conjugated rabbit F(ab')<sub>2</sub> anti-mouse IgG antibody (0.4 mg/ml, F/P = 2.4). Cells were sorted at a rate of 2,800-3,000 cells per s, with the scatter gates for fluorescence set to remove the IgD-positive cells (5).

**Separation of B Cells on Density Gradients.** B cell-enriched fractions were prepared by rosetting adherent cell-depleted MNC with aminoethylisouronium bromide hydrobromide (AET)-treated sheep erythrocytes (SRBC) followed by separation on Ficoll-Hypaque density gradients (8). The non-rosette-forming fraction (B cell fraction) contained <3% cells reactive with the pan-T antibody, OKT3 (Ortho Pharmaceutical, Raritan, NJ). B cells were further separated according to their densities using discontinuous gradients of Percoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Uppsala, Sweden) as described (8). Briefly, 0.5 ml of B cell suspension ( $20-60 \times 10^6$  cells/ml) in 30% Percoll in PBS was layered onto discontinuous gradients ranging from 45 to 60% Percoll in 5% increments of 2.5 ml in 15-ml centrifuge tubes. Gradients were centrifuged at 3,000 g for 5 min at 4°C.

**Rosette Formation with Mouse Erythrocytes.** B cells were examined for rosette formation with mouse erythrocytes (MRBC) as described (9). Rosettes were prepared by mixing 50  $\mu$ l of the B cell fraction ( $2-4 \times 10^6$  cells/ml), 100  $\mu$ l of 1% neuraminidase-treated MRBC, and 50  $\mu$ l of FCS absorbed with MRBC. Cells were preincubated for 15 min at 37°C and pelleted by centrifugation at 1,000 rpm for 5 min. After overnight incubation at 4°C, cells were resuspended gently, and rosette formation was examined. A lymphocyte with three or more MRBC attached was counted as a positive rosette.

**Immunofluorescence Studies.** Affinity-purified and fluorochrome-conjugated goat antibodies specific for human  $\mu$ ,  $\delta$ ,  $\gamma$ ,  $\alpha$ ,  $\kappa$ , and  $\lambda$  chains were used to enumerate sIg isotypes on B cells, as described (5, 10). At least 500 cells in each sample were counted using a Leitz fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ).

**Enumeration of Cytoplasmic (c) Ig-positive Cells.** The numbers of plasma cells were estimated in PWM-stimulated cell cultures maintained for 7 d. Cell viability, estimated by counting harvested cells that excluded trypan blue dye, ranged from 60 to 80%. The cells were spun onto glass slides by cytocentrifugation, fixed in 95% ethanol and 5% acetic acid at -20°C, washed in PBS, and stained with either fluorescein- or rhodamine-conjugated goat anti-human  $\mu$ ,  $\gamma$ , or  $\alpha$  antibodies, as described (5). The percentage of cells brightly stained for cytoplasmic Ig determinants was determined using incidence illumination immunofluorescence microscopy. At least 1,000 cells were counted for each isotype, and the absolute number of cIg<sup>+</sup> cells per culture well was calculated by multiplying the percentage of cIg<sup>+</sup> cells by the number of viable cells.

**Measurement of Cell Size.** Cell diameters of 100 sIgM<sup>+</sup>sIgD<sup>+</sup> and 100 sIgM<sup>+</sup>sIgD<sup>-</sup> cells were determined using a calibrated microscope ocular after spinning immunofluorescence staining cells onto glass slides by cytocentrifugation.

## Results

**Effect of Depletion of SIgD<sup>+</sup> Cells on PWM-induced Plasma Cell Differentiation.** To examine whether B cells responding to PWM stimulation bear IgD, blood MNC

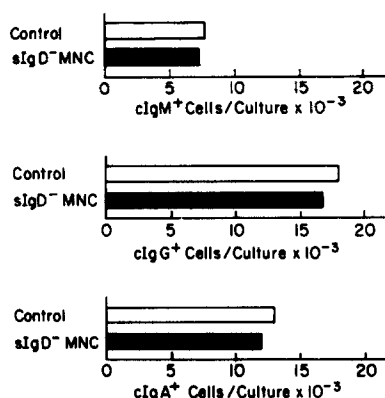


FIG. 1. Effect of depletion of sIgD<sup>+</sup> lymphocytes on PWM-induced plasma cell differentiation. Blood MNC were depleted of sIgD<sup>+</sup> cells by the panning method, as described in Materials and Methods. Control and sIgD<sup>-</sup>-depleted (sIgD<sup>-</sup>) MNC were cultured with PWM for 7 d. Data are from one of three reproducible experiments.

TABLE I  
*Surface Ig Isotypes Expressed by B Cells Remaining in Blood MNC Preparations Selectively Depleted of IgD<sup>+</sup> Cells*

Experiment	Separation procedure	Mononuclear cells	Percent positive cells			
			sIgM	sIgD	sIgG	sIgA
I	Panning*	Control	9.9	9.2	2.5	1.7
		sIgD <sup>-</sup>	1.5	0.2	2.1	1.6
II	FACS‡	Untreated	9.4	8.9	1.5	1.1
		Unsorted§	9.3	8.9	1.5	1.1
		sIgD <sup>-</sup>	0.4	<0.1	1.1	0.8

\* MNC were treated with monoclonal anti- $\delta$  antibody or PBS alone, and sIgD<sup>+</sup> cells were depleted by adherence to rabbit anti-mouse IgG antibody-coated dishes.

‡ MNC were treated with monoclonal anti- $\delta$  antibody, stained with FITC-rabbit F(ab')<sub>2</sub> anti-mouse IgG antibody, and sorted with a FACS.

§ MNC were incubated with antibody but not sorted.

preparations were selectively depleted of sIgD<sup>+</sup> cells by the "panning" method and then stimulated with PWM for 7 d. The sIgD<sup>+</sup>-depleted (sIgD<sup>-</sup>) MNC gave rise to numbers of IgM-, IgG-, and IgA-producing cells comparable to those obtained in control MNC samples (Fig. 1), although the frequency of sIgD<sup>+</sup> cells was reduced from 9.2 to 0.2% and of sIgM<sup>+</sup> cells from 9.9 to 1.5% (Table I). This striking result was confirmed in an experiment in which sIgD<sup>+</sup> cells were selectively depleted using the FACS (Fig. 2). The number of cIg<sup>+</sup> cells was only slightly reduced in the sIgD<sup>+</sup>-depleted MNC culture as compared with the untreated control, and this difference could be attributable to the reduced recovery of viable cells in cultures of the sIgD<sup>+</sup>-depleted population and the anti- $\delta$  treated, unsorted control (85 and 87% of the untreated control, respectively). The completeness of the sIgD<sup>+</sup> cell depletion in this experiment was confirmed by surface immunofluorescence staining of the cells before culture (Table I) and by estimating cIgD<sup>+</sup> cells at the end of the culture interval (Fig. 2). When MNC were cultured with PWM, a small population of IgD plasma cells was induced, but cIgD<sup>+</sup> cells were absent in the sIgD<sup>+</sup>-depleted culture.

In agreement with previous studies (6, 7), anti- $\delta$  appeared to enhance PWM-induced IgM plasma cell differentiation in this experiment (Fig. 2), and we have

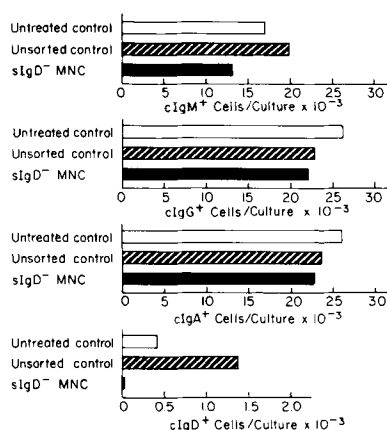


FIG. 2. Effect of depletion of sIgD<sup>+</sup> lymphocytes on PWM-induced plasma cell differentiation. Blood MNC were depleted of sIgD<sup>+</sup> cells by the FACS, as described in Materials and Methods. Untreated control, labeled but unsorted control, and sIgD<sup>+</sup>-depleted (sIgD<sup>-</sup>) MNC were cultured with PWM for 7 d.

analyzed the mechanism of this enhancement in other experiments (to be published). This positive effect of anti- $\delta$  antibody treatment mitigated against its use in purifying sIgD<sup>+</sup> cells to examine further their responsiveness to PWM alone.

*Expression of Surface Ig Isotypes and Mouse Erythrocyte Receptors by Subpopulations of B Cells Separated on Density Gradients.* The results of two previous experiments in our laboratory appear directly relevant to the observations described above. Rosette formation with mouse erythrocytes defined a subpopulation of human B cells (MRFC<sup>+</sup> fraction) positive for sIgM and sIgD, which responded very poorly to PWM stimulation as compared with an MRFC<sup>-</sup> fraction containing sIgM<sup>+</sup>sIgD<sup>-</sup> cells (9). In another study, B cells of relatively low buoyant density were most efficiently induced by PWM to differentiate into plasma cells, whereas B cells of high density gave the best plasma cell response to lipopolysaccharide (8). We confirmed these results (data not shown) and then examined the sIg isotypes and the capacity for rosette formation with mouse erythrocytes exhibited by B cells fractionated by centrifugation on discontinuous Percoll gradients. As shown in Table II, B cells of relatively low density (Fx-1), which could respond to PWM very well, included the highest proportion of sIgM<sup>+</sup>sIgD<sup>-</sup> cells. More than 95% of sIgM<sup>+</sup> cells in the higher density bands (Fx-2 and Fx-3) expressed sIgD. IgG and IgA B cells, many of which lack detectable sIgD (Table I, ref. 10), were greatly enriched in fraction 1. B cells lacking receptors for mouse erythrocytes also preferentially sedimented in this low density fraction (Table II).

*Relative Cell Size of sIgM<sup>+</sup>sIgD<sup>+</sup> and sIgM<sup>+</sup>sIgD<sup>-</sup> Cells.* MNC were stained with both rhodamine-labeled anti- $\mu$  and fluorescein-labeled anti- $\delta$  antibodies and examined by immunofluorescence microscopy after centrifugation onto glass slides. The mean and range of cell diameters of sIgM<sup>+</sup>sIgD<sup>+</sup> and sIgM<sup>+</sup>sIgD<sup>-</sup> cells were 12.6 (10.4–16.0)  $\mu$ m and 14.8 (11.2–19.2)  $\mu$ m, respectively. Thus, the sIgD<sup>-</sup> B cells appeared significantly larger than sIgD<sup>+</sup> cells ( $P < 0.01$ ).

### Discussion

Distinct subpopulations of B lymphocytes with differing requirements for activation may appear sequentially and express different sets of sIg during development. Surface

TABLE II  
*Distribution of Surface Ig Isotypes and Mouse Erythrocyte Receptors on B Cells Separated by Density Gradient Centrifugation*

Experiment	B cell fraction* (Percoll Concentration)	Percent positive cells‡						Percent sIgM <sup>+</sup> sIgD <sup>-</sup> / sIgM <sup>+</sup>	Percent MRFC <sup>+</sup> / sIg <sup>+</sup>
		sIg	sIgM	sIgD	sIgG	sIgA	MRFC		
I	Total	51.5	47.9	44.8	4.0	3.1	37.6	7.0	73.0
	Fx-1(45/50)	29.4	23.5	20.2	4.8	4.0	6.5	14.1	22.1
	Fx-2(50/55)	75.8	72.4	71.3	3.8	2.3	42.7	3.8	56.3
	Fx-3(55/60)	96.4	94.8	95.3	0.6	0.2	71.1	1.3	73.8
II	Total	42.4	39.2	36.1	7.3	4.1	36.4	8.0	85.8
	Fx-1(45/50)	30.5	21.5	17.3	4.1	4.9	7.0	23.5	23.0
	Fx-2(50/55)	66.5	65.2	63.6	2.8	3.1	40.0	5.1	60.2
	Fx-3(55/60)	72.4	69.6	70.6	1.4	0.9	N.D.§	0.7	N.D.

\* B cells were enriched from blood MNC preparations by removal of E rosette-forming T cells and then separated further by centrifugation on discontinuous Percoll gradients. Cells banding at each interface between Percoll densities of 45 and 50%, 50 and 55%, and 55 and 60% were collected.

‡ Surface Ig were detected by immunofluorescence and mouse erythrocyte receptors by a rosetting procedure, as described in Materials and Methods.

§ Not done.

IgD is acquired after sIgM expression during B cell maturation and expressed together with sIgM on the majority of B cells in human (10, 11). Surface IgD is then lost after antigen or mitogen stimulation (12–14) and during the maturation of memory B cells (12).

Recent studies in mice suggest that small resting B cells are triggered by antigen and Ia-complementary specific helper T cells, by mitogens, or by anti-Ig antibodies and transformed into large excited or activated B cells. The latter exhibit responsiveness in a non-MHC-restricted fashion to T cell factors that induce polyclonal B cell replication and maturation (15).

Our results suggest that human B cells responsive to PWM are relatively large and of low density, lack surface receptors for mouse erythrocytes, and, except for a few IgD plasma cell precursors, have little or no sIgD. This PWM-inducible subpopulation of sIgD<sup>-</sup> B cells can be further divided into sIgM<sup>+</sup> plasma cell precursors and committed sIgG<sup>+</sup> and sIgA<sup>+</sup> precursors that may or may not express sIgM (5). Placing these results within the context of the information discussed above, we propose a differentiation scheme in which PWM-responsive B cells represent a distinctive subpopulation of relatively mature and preactivated sIgD<sup>-</sup> cells.

### Summary

Surface immunoglobulin D (IgD)-positive lymphocytes precoated with monoclonal anti- $\delta$  antibody were selectively removed from blood mononuclear cell preparations by "panning" and by fluorescence-activated cell sorter. The depletion of sIgD<sup>+</sup> cells did not significantly affect plasma cell responses to pokeweed mitogen (PWM). PWM-responsive B cells lacking sIgD and mouse erythrocyte receptors preferentially sedimented in lower density fractions of a discontinuous Percoll gradient, and sIgD-negative B cells were found to have a larger mean diameter than IgD-positive cells. We conclude that PWM-responsive B cells represent a distinct subpopulation of relatively large cells that have ceased to express receptors for mouse erythrocytes and surface IgD.

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